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Molecular Phylogeny of Zooxanthellate Bivalves

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Abstract. The aim of this research was to analyze the phylogenetic relationships of zooxanthellate bivalves belonging to the genera *Tridacna, Hippopus, Fragum,* and *Corculum* as well as to the closely related azooxanthellate bivalves belonging to *Vasticardium* and *Fulvia.* The small-subunit ribosomal RNA genes (18S rDNAs) from these bivalves were amplified by polymerase chain reaction with universal eukaryotic primers and were then sequenced. The sequence data from each species were analyzed by the neighbor-joining, maximum parsimony, and maximum likelihood methods, and phylogenetic trees were constructed. The results were essentially consistent with the morphological taxonomy of these bivalves. Thus, the zooxanthellate clams branch into two lineages, one composed of the genera *Fragum* and *Corculum* in the family Cardiidae, and the other composed of the genera *Tridacna* and *Hippopus* in the family Tridacnidae. However, present results indicate that the azooxanthellate clams analyzed (*Vasticardium flavum* and *Fulvia mutica*) are more likely to form a clade with the species of *Tridacna* and *Hippopus* than with those of *Fragum* and *Corculum.* This topology suggests that either the symbiosis with zooxanthellae occurred independently in each of two lineages, *Tridacna-Hippopus* and *Corculum-Fragum,* or the symbiosis was established in clams ancestral to the lineages of both the zooxanthellate clams and the azooxanthellate clams *Vasticardium* and *Fulvia,* and the latter lost the symbiotic relationship after the symbiotic clam lineages had diverged.

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Introduction

Symbioses between marine invertebrates and microalgae have been known for years (Smith and Douglas, 1987; Trench, 1993). Although various microalgae—from cyanobacteria to diverse eukaryotic species—are known as symbionts, zooxanthellae (a common name for symbiotic dinoflagellates) dominate marine invertebrate symbioses, including those in corals, molluscs, flatworms, and protozoa. In bivalves, symbioses with microalgae are found in two distinct groups: freshwater clams in the genus *Anodonta* (Unionidae) that harbor a symbiotic green alga (zoochlorella) (Goetsch and Scheuring, 1926; Pardy, 1980); and the giant clams and related clams that harbor zooxanthellae. The giant clams belong to the genera *Tridacna* and *Hippopus* (Rosewater, 1965, 1982; Yonge, 1980) in the family Tridacnidae. Less well known clams harboring zooxanthellae belong to the genera *Corculum* (Kawaguti, 1950) and *Fragum* (Kawaguti, 1983a; Ohno *et al.,* 1995) in the family Cardiidae. No bivalve in any other family has been reported to have a symbiotic relationship with zooxanthellae.

Zooxanthellae are thought essential to the clams that contain them. Photosynthetic products secreted from the zooxanthellae are taken up by the giant clam and contribute nutrition (Fisher *et al.,* 1985; Griffiths and Streamer 1988; Klumpp *et al.,* 1992). No adult giant clam devoid of zooxanthellae has ever been found, though a partially albino *T. gigas* has been reported (Yellowlees *et al.,* 1993; Norton *et al.,* 1995). The zooxanthellae also benefit from the association: they lack UV-absorbing substances and are sensitive to UV light, but are protected by the mantle tissue of the host clam (Ishikura *et al.,* 1997).

The evolution of the symbiosis between the zooxanthel-

lae and the clams has been enigmatic. Recently, however, the methods of molecular phylogeny have been applied to the relationship between zooxanthellae and various marine invertebrate hosts (Rowan and Powers, 1991, 1992; MacNally *et al.*, 1994). These reports have shown that symbiotic dinoflagellates from various hosts are diverse and that phylogenetic similarity among the dinoflagellates is not correlated with the phylogenetic affiliations of their hosts. These data support the concept that the symbioses between dinoflagellates and marine invertebrates are polyphyletic (Trench, 1992), but the phylogeny of the giant clams has not yet been subjected to molecular analysis.

Symbiosis with zooxanthellae occurs only in two closely related families of bivalves, Tridacnidae and Cardiidae (Ohno *et al.*, 1995). Although all of the eight living Tridacnidae clams contain zooxanthellae (Norton and Jones, 1992), this relationship occurs in only a few species in two genera of Cardiidae (Ohno *et al.*, 1995). To clarify the phylogenetic relationship of these zooxanthellate and azooxanthellate bivalves, we analyzed the 18S rDNA sequences from seven zooxanthellate tridacnid clams (five species of *Tridacna* and two of *Hippopus*), three zooxanthellate cardiid clams (one species of *Corculum* and two of *Fragum*), and two azooxanthellate cardiid clams (one species each of *Fulvia* and *Vasticardium*).

Materials and Methods

Collection of clam specimens

Tridacnid clams (*Tridacna maxima, T. squamosa,* and *Hippopus hippopus*), zooxanthellate cardiid clams (*Fragum fragum, F. unedo*), and an azooxanthellate cardiid clam (*Vasticardium flavum*) were collected in the Republic of Palau, Western Caroline Islands, during the cruises of the R/V *Sohgen-maru* in 1993–1994. *T. gigas, T. derasa,* and *H. porcellanus* were purchased from the PMDC (Palau Mariculture Demonstration Center). Live specimens of a zooxanthellate cardiid, *Corculum cardissa,* which had been collected from mariculture tanks for tridacnid clams, were gifts from PMDC. A live specimen of *Fragum fragum* collected in Okinawa, Japan, was a gift from Mr. Osumi of Ryukyus University. An azooxanthellate cardiid clam, *Fulvia mutica,* was purchased from a local fishery market in Shimizu, Japan. The shells were saved (except for those of *T. gigas,* which were lost during transportation) and were kindly identified by Prof. Okutani of Nihon University.

Extraction of DNA from the clams

Live clams were dissected, and the organs were separated and frozen at −20°C until used. The white part of the gill or foot tissue (0.1–0.2 g), which was devoid of zooxanthellae, was homogenized in a glass homogenizer in 2–3 ml TE buffer (10 m*M* Tris-HCl, 100 m*M* EDTA, pH 8.0) containing 0.5% sodium dodecyl sulfate, and digested with proteinase K (100 μg/ml) at 50°C for 3 h (Wada *et al.*, 1992). The DNA was then extracted with TE buffer-saturated phenol and washed twice with a mixture of chloroform and isoamyl alcohol (24:1). An equal volume of 5 *M* ammonium acetate was then added, and the DNA was precipitated with cold ethanol, washed once with cold 70% ethanol, and dried.

Amplification of 18S rDNA and DNA sequence determination

A polymerase chain reaction (PCR) kit (Takara Shuzo, Kyoto, Japan) was used to amplify the 18S rDNA from 1 μg of genomic DNA. The manufacturer's instructions were followed; *i.e.*, 30 cycles comprising 93°C for 1.5 min, 58°C for 1.5 min, and 72°C for 2 min. The extension reaction at 72°C in the final cycle was prolonged to 10 min, and the PCR products were then frozen at −20°C until used. The universal eukaryotic primers used in the amplification of the 18S-rDNA were 5′-GGTTGATCCTGCCAGTAGTCATATGCTTG-3′ (ss5) and 5′-GATCCTTCCGCAGGTTCACCTACGGAAACC-3′ (ss3). These sequences were reported to be located four nucleotides from the 5′ and 3′ ends, respectively, of the 18S-rRNA of *Prorocentrum micans,* a dinoflagellate (Herzog and Maroteaux, 1986; Rowan and Powers, 1992). Amplified DNAs were cloned in pT7 plasmids (Novagene, USA) with *E. coli* JM 109 as the host. At least three cloned DNAs amplified from the genomic DNA of an individual clam were sequenced for each clam species, using custom-made 20-b DNA primers (Japan BioService, Saitama), a Takara PCR sequence kit with an ABI-type 373A DNA sequencer.

Analysis of the sequence data

The 18S rDNA sequences were aligned using Clustal W (Thompson *et al.*, 1994) and gap and insertion regions removed with the software program RMINSDEL in MOLPHY-2.2 (Adachi and Hasegawa, 1994). The aligned sequences were then analyzed by the neighbor-joining (NJ) method (Saitou and Nei, 1987) using the DNADIST and NEIGHBOR programs (using the Kimura 2-parameter model; Kimura, 1980) in PHYLIP 3.57c (Felsenstein, 1995). The statistical significance of each cluster in the tree was evaluated with 1000 iterations of bootstrap resamplings and tree reconstructions (Felsenstein, 1985) using DNADIST, NEIGHBOR, SEQBOOT, and CONSENSE in PHYLIP 3.57c. The same sequences were analyzed by the maximum parsimony

(MP) method using the DNAPARS program in PHYLIP 3.57c. Decay analysis (Bremer, 1988, 1994; Winnepenninckx *et al.*, 1996) was also undertaken by using PHYLIP 3.57c. The sequence data of nine selected clams were further analyzed by the maximum likelihood (ML) method using the NUCML program adopting the Alpha/Beta ratio 2.0 model (Hasegawa *et al.*, 1985) in MOLPHY 2.2 (Adachi and Hasegawa, 1994). The tree with minimal AIC (Akaike Information Criterion), which is defined as −2 (log-likelihood) + 2 (number of free parameters) (Akaike, 1974), was considered the most appropriate tree. The statistical significance of each cluster in the ML tree was evaluated by the bootstrap analysis with 1000 iterations. Phylogenetic trees were drawn with TreeView (Page, 1996) on a Macintosh computer.

Results

The PCR products

Lengths of the amplified 18S rDNAs (between the primers ss5 and ss3) from the zooxanthellate and azooxanthellate cardiid clams were 1780–1875 bp—longer than the corresponding lengths in the other clams examined (1745–1772 bp) (Table 1). Those sequences were deposited in the DDBJ (DNA data base of Japan) under the accession numbers shown in Table 1. The clam shells (except *T. gigas*) were deposited in The National Science Museum in Tokyo with the specimen numbers listed in Table 1.

Phylogenetic trees

The alignment of 18S rDNA sequences of the clams is available on the web site of this journal at the following address:

http://www.mbl.edu/html/BB/home.BB.html

After removing the gaps and insertions, we analyzed 1698 sites within the sequences by the NJ and MP methods. Figure 1 shows a phylogenetic tree constructed by the NJ method. The 18S rDNA sequence of *Limicolaria kambeul* (Gastropoda, Pulmonata, Achatinidae) was chosen as the outgroup. We also analyzed the data with the 18S rDNA

Table 1

Length of amplified 18S-rDNAs

Species	Place of collection	Length of the PCR products[1]	Accession number[2]	Specimen number[3]
Zooxanthellate bivalves (present work)				
Tridacna gigas	Palau	1780 bp	D84189	n.d.
Tridacna derasa	Palau	1803	D84658	NSMT-Mo70918
Tridacna crocea	Okinawa (Japan)	1803	D88908	NSMT-Mo70917
Tridacna maxima	Palau	1805	D84659	NSMT-Mo70919
Tridacna squamosa	Palau	1804	D84190	NSMT-Mo70916
Hippopus hippopus	Palau	1813	D84660	NSMT-Mo70914
Hippopus porcellanus	Palau	1813	D84661	NSMT-Mo70915
Fragum fragum	Palau	1865	D84662	NSMT-Mo70921
Fragum fragum	Okinawa (Japan)	1865	D84663	NSMT-Mo70920
Fragum unedo	Palau	1865	D84664	NSMT-Mo70922
Corculum cardissa	Palau	1875	D88909	NSMT-Mo70923
Azooxanthellate Cardiidae bivalves (present work)				
Vasticardium flavum	Palau	1852	D88910	NSMT-Mo70913
Fulvia mutica	Mikawa-bay (Japan)	1840	D88911	NSMT-Mo70912
Other bivalves (from database)				
Spisula solidissima		1772	L11270	
Tresus nuttali		1772	L11269	
Placopecten magellanicus		1745	X53899	
Chlamys islandica		1746	L11232	
Mytilus edulis		1757	L24489	
Gastropod (from database)				
Limicolaria kambeul		1770	X66374	

[1] Length of the amplified DNA between but excluding primers ss5 and ss3.

[2] Accession number of the DNA sequence data in DNA Data Base of Japan.

[3] Deposit number of the shell specimen in National Science Museum, Tokyo; n.d., not deposited.

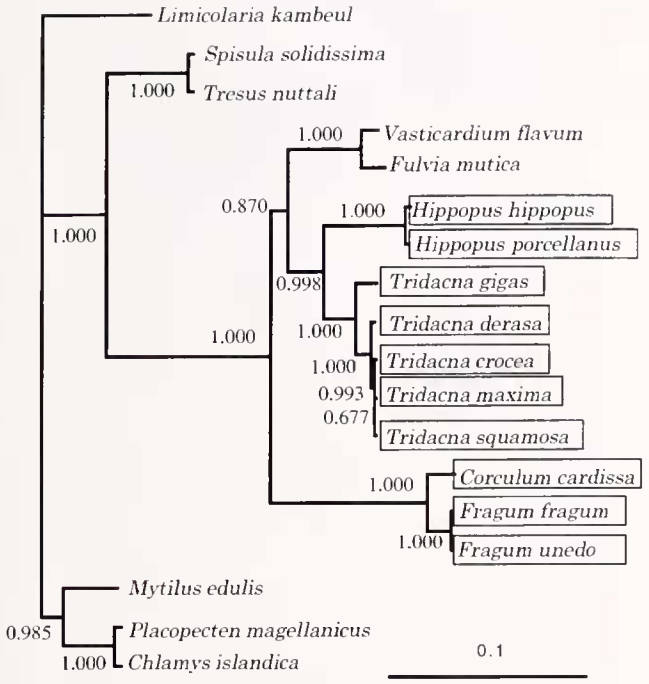

Figure 1. A phylogenetic tree of zooxanthellate and azooxanthellate clams, calculated by the neighbor-joining method adopting the Kimura 2-parameter model. Numbers at the nodes are the bootstrap values for the clades in 1000 replications. Box, zooxanthellate clam. Bar, 0.10 substitutions per site.

sequence of *Symbiodinium corculorum*, the symbiotic alga of *Corculum cardissa* (accession number L13717) as the outgroup; the results were the same as those with *L. kambeul* (data not shown). Clams belonging to the Pteriomorphia (*Mytilus edulis* in Mytilidae, and *Placopecten magellanicus* and *Chlamys islandica* in Pectinidae) made a clade that was clearly separated from those of the Heterodonta. In the heterodont branch, a clade including the families Tridacnidae and Cardiidae—and comprising all of the zooxanthellate clams examined, as well as two azooxanthellate clams, *V. flavum* and *F. mutica*—was clearly separated from another clade containing two species in the family Mactridae (*Spisula solidissima* and *Tresus nuttali*). This clade was further resolved into three lineages: *Corculum-Fragum*, *Vasticardium-Fulvia*, and *Tridacna-Hippopus*. The NJ tree indicates that the zooxanthellate cardiids and azooxanthellate cardiids diverged before the zooxanthellate tridacnids. The bootstrap value at the node uniting the *Vasticardium-Fulvia* and *Tridacna-Hippopus* clades was 0.870, and that at the node uniting the *Vasticardium-Fulvia-Tridacna-Hippopus* and *Corculum-Fragum* clade was 1.000. This indicates that tridacnid clams are more likely to form a clade with clams of *Vasticardium-Fulvia* than with those of *Fragum-Corculum*. Three tridacnid clams, *T. maxima*, *T. squamosa*, *and T. crocea*, are evidently very closely related, whereas *T. gigas* and *T. derasa* are relatively distant from them. *Fragum fragum* and *F. unedo* are very closely related, the identity between their 18S rDNA sequences being 99.8% (1861 out of 1865 bp).

The maximum parsimony (MP) method gave three equally most parsimonious trees of similar, but distinct, topologies (Fig. 2, A–C). The lengths of the trees were

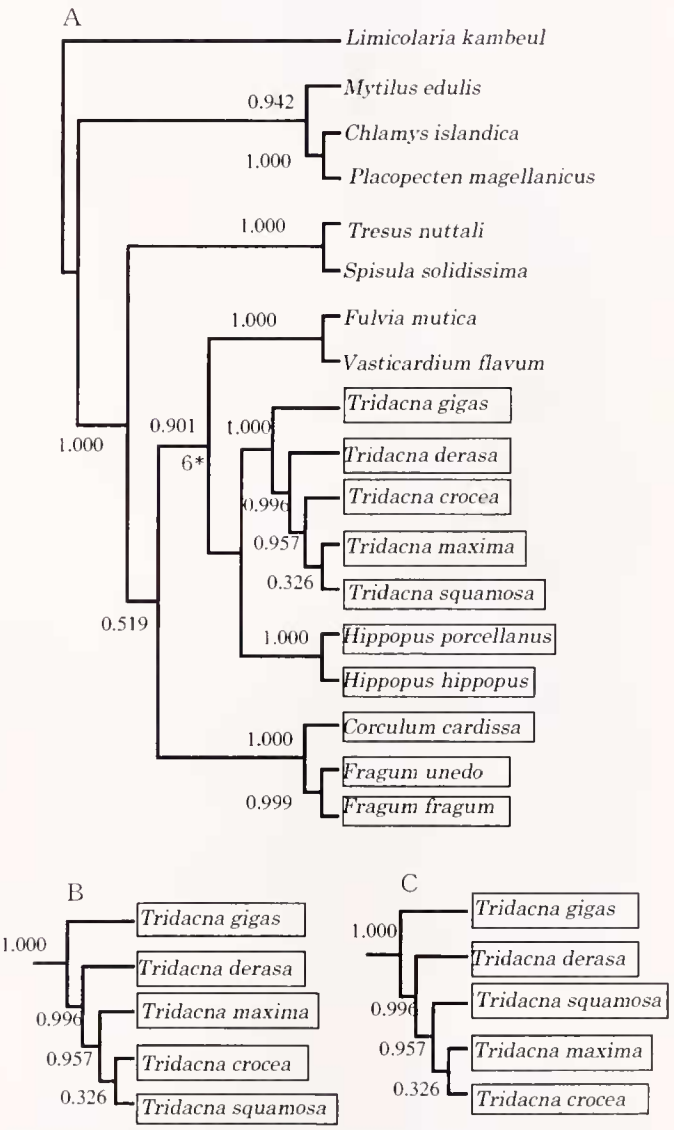

Figure 2. Phylogenetic trees of zooxanthellate and azooxanthellate clams, calculated by the maximum parsimony method. Three topologies were selected as the best tree; the differences were found in *Tridacna squamosa*, *T. crocea*, and *T. maxima*. (A) One of the best trees. (B and C) Parts of the two other trees showing the dissimilarities to A. Numbers at the nodes are the bootstrap values for the clades in 1000 reptications. *, decay index. Box, zooxanthellate clam. Consistency index, 0.76; retention index, 0.88.

1019 steps. Consistency and retention indices of the three trees were 0.76 and 0.88, respectively. These trees had essentially the same topology as that obtained by the NJ method, except for the positions of the three closely related clams in the genus *Tridacna* (*T. squamosa, T. crocea,* and *T. maxima*). These differences suggest that the three species are closely related. In this tree, bootstrap value for the clade containing the tridacnid clams and *Vasticardium-Fulvia* was high (0.901). The decay index, which is the number of extra steps required before this clade collapsed, was calculated to be 6. These data suggest that the azooxanthellate cardiid lineage is more likely to form a clade with the zooxanthellate tridacnid clam lineage than with the zooxanthellate cardiid clam lineage. Lineages *Tridacna-Hippopus-Vasticardium-Fulvia* and *Corculum-Fragum* formed a clade, but the bootstrap value was relatively low (0.519).

Due to the computational limitations, we selected nine clam species for a maximum likelihood analysis: one from subclass Pteriomorphia (*Mytilus edulis*) as an outgroup, two from zooxanthellate Cardiidae (*Corculum cardissa* and *Fragum fragum*), two from azooxanthellate Cardiidae (*Vasticardium flavum* and *Fulvia mutica*), two from the genus *Tridacna* (*Tridacna gigas* and *T. derasa*), and two from *Hippopus* (*H. hippopus* and *H. porcellanus*). A total of 1713 sites were used for the ML analysis. The tree topology (Fig. 3), was essentially the same as that obtained by the NJ method. The bootstrap value at the node of *Tridacna-Hippopus* and *Vasticardium-Fulvia* was 0.76, and that of *Tridacna-Hippopus-Vasticardium-Fulvia* and *Corculum-Fragum* was 1.00. The differences between the minimal AIC (Akaike Information Criterion, as defined in the Materials and Methods) and the AICs of the other possible trees were greater than 15.5 ($>>1.0$), which suggests that the tree topology shown in Figure 3 is statistically significant.

To examine the genetic difference between individuals from two distant locations, 18S rDNA sequences of *F. fragum* collected from Okinawa and Palau were compared. The identity between them, 99.9% (1864 out of 1865 bp), indicated no significant difference between them.

Insertions and gaps

Table II shows the major gaps and insertions found in 18S rDNAs of cardiids and tridacnids when aligned with those of other clams, especially *Limicolaria kambeul*, listed in Table I. Designations of the regions of the 18S rRNA are shown according to Winnepennickx *et al.* (1992). The table includes any insertions and gaps longer than 10 b in any of the cardiids and tridacnids. Corresponding smaller gaps and insertions in other zooxanthellate clams are also shown. Insertions in the E10-1 region are restricted to the azooxanthellate cardiid calms, *V. flavum* and *F. mutica*. On the other hand, zooxanthellate clams have gaps in this region. Some of those insertions were aligned and are shown in Figure 4. The identity between E10-1 insertions of *V. flavum* and *F. mutica* is low (70%). While the inserts in *Corculum* and *Fragum* are similar to each other, those in *Hippopus* are different from those of *Corculum* and *Fragum*. Inserts in region 47 are restricted to *Tridacna*, but *T. gigas* lacks this insertion. Those of *T. squamosa* and *T. maxima* are the same: one base different from that of *T. crocea*, and two bases different from that of *T. derasa*.

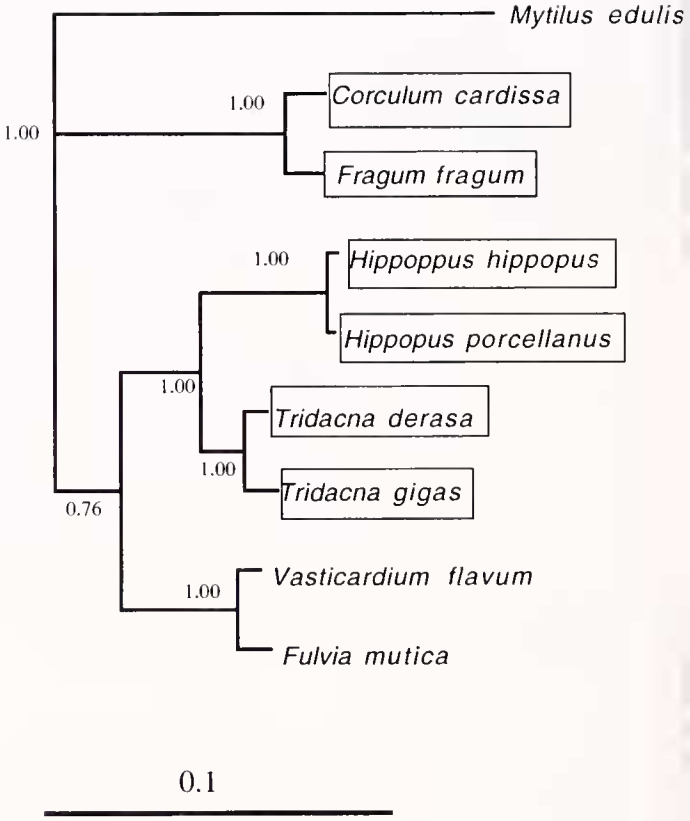

Figure 3. A phylogenetic tree of nine selected bivalves, including zooxanthellate and azooxanthellate clams, calculated by the maximum likelihood method. Bar indicates 0.1 base substitutions per site. Numbers at the nodes are the bootstrap values for the clades in 1000 replications. Box, zooxanthellate clam.

Discussion

The tree topologies based on 18S rDNA sequences (Figs. 1–2) are consistent with the morphological taxonomy of Mytilidae, Pectinidae, and Mactridae (Morton, 1996). The family Tridacnidae comprises two genera, *Tridacna* and *Hippopus* (Rosewater, 1965, 1982). The genus

Table II

Major insertions and gaps in 18S rDNAs in tridacnid and cardiid clams in comparison with those of Limicolaria kambeul

	Regions*	Positions in the alignment†	Size (b)	Species‡
Insertions				
	E10-1	247-269	23	Vf
			22	Fm
	E21-2	768-782	15	Cc
			12	Ff, Fu
			11	Hh, Hp
			4	Vf, Fm
			3	Tg
	41	1484-1513	30	Ff, Fu
			27	Cc
			4	Vf, Fm, Hh, Hp, Tg, Td, Tc, Ts, Tm
	47	1879-1910	32	Ts, Tc, Tm
			31	Td
			8	Cc
			6	Fm
			5	Fu, Ff, Vf, Tg
Gaps				
	E10-1	232-281	25§	Hh, Hp, Tg, Td, Tc, Ts, Tm
		232-246	14	Cc, Fu, Ff

Gaps and insertions were detected in the aligned 18S rDNAs listed in Table 1.

* Regions in 18S rRNA of *Limicolaria kambeul* designated in Winnepennickx *et al.* (1992).

† The alignment is available at the following URL:
http://www.mbl.edu/html/BB/home.BB.html

‡ Cc, *Corculum cardissa;* Fu, *Fragum unedo;* Ff, *Fragum fragum;* Vf, *Vasticardium flavum;* Fm, *Fulvia mutica;* Hh, *Hippopus hippopus;* Hp, *Hippopus porcellanus;* Tg, *Tridacna gigas;* Td, *Tridacna derasa;* Tc, *Tridacna crocea;* Ts, *Tridacna squamosa;* Tm, *Tridacna maxima.*

§ Very close gaps or insertions were combined.

Tridacna is further divided into three subgenera, *Tridacna, Persikima,* and *Chametrachea. Tridacna gigas* belongs to subgenus *Tridacna; T. derasa* to *Persikima;* and three species, *T. squamosa, T. maxima,* and *T. crocea,* belong to *Chametrachea.* This classical taxonomy also agrees well with the present tree topologies (Figs. 1–3). However, the present data are also in marked disagreement with the classical taxonomy: *i.e.,* the tridacnid clams, genera *Tridacna* and *Hippopus,* are more closely related to the cardiids *Vasticardium flavum* and *Fulvia mutica* than these clams of *Fragum* and *Corculum,* suggesting either that the family Cardiidae is paraphyletic or that tridacnids belong to the family Cardiidae. The correct interpretation—either the traditional taxonomy of Tridacnidae and Cardiidae or the present molecular view of these groups—is obscure. Molecular phylogenetic analyses using some other genes are underway and may answer this question.

The present results indicate that the divergence between the *Corculum-Fragum* lineage and that of *Vasticardium-Fulvia* is deeper than that between *Corculum-Fragum* and *Tridacna-Hippopus.* Because there is no evidence of symbiosis in clams of *Vasticardium* and *Fulvia,* clams in the tridacnid and *Corculum-Fragum* lineages might have acquired symbiotic relationships with zooxanthellae independently after their divergence from the lineage of *Vasticardium-Fulvia.* An alternative explanation is that the clam ancestral to the three lineages acquired the symbiotic relationship with zooxanthellae, and only the lineage of azooxanthellate Cardiidae lost this characteristic. If the latter explanation is correct, there may be some traces of symbiosis, not yet reported, in some of the azooxanthellate Cardiidae. Further studies are necessary to distinguish between these two hypotheses.

Masuda *et al.* (1994) reported that extracts of mantle homogenates from tridacnid clams (*Tridacna derasa, T. maxima, T. crocea,* and *H. hippopus*) are much more stimulatory to the excretion of photosynthate by *T. derasa* zooxanthellae than are tissue extracts of zooxanthellate *Fragum* clams, other azooxanthellate bivalves, or gastropods. These may reflect the relative phylogenetic distance between tridacnids and zooxanthellate cardiids, although the active substance in the mantle homogenate is still not known.

Tridacna crocea larvae develop through several morphological stages: straight-hinge and prodissoconch,

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Inserts in E10-1 region
             247                          269
              |                            |
V. flavum  1:T-TG-TTTCAGCGTCCGCAGG-GCGG
F. mutica  1:TCCGCTCTC-GGGT-GGC-GGCCCCG
             *    * ** * **  ** **  * *

Inserts in E21-2 region
                    768             782
                     |               |
C. cardissa       1:TCCT-TACTAG-CTCCG
F. unedo          1:-CC----CTTGCCTCCG
F. fragum         1:-CC----CTTGCCTCCG
H. hippopus       1:-CCTGTA---G-C-AAA
H. porcellanus    1:-CCTGTA---G-C-AAA
                     **       * *

Inserts in 41 region
                 1484                                1513
                  |                                   |
C. cardissa   1:GGGACCCAGG---GACCGCTCT-CGAGCGGA
F. fragum     1:GGGACCTAGGTCCGTCCGCTCTGCGGG-GGA
F. unedo      1:GGGACCTAGGTCCGTCCGCTCTGCGGG-GGA
                ****** ***   * ******* ** * ***

Inserts in 47 region
                  1879                              1910
                   |                                 |
T. derasa      1:GTTG-GTTTCATCTCCTCGCGGGGTGTGCCTT
T. crocea      1:GTTGCGCTCCATCTCCTCGCGGGGTGGGCCTT
T. maxima      1:GTTGCGCTTCATCTCCTCGCGGGGTGGGCCTT
T. squamosa    1:GTTGCGCTTCATCTCCTCGCGGGGTGGGCCTT
                 **** * * ***************** *****
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Figure 4. Inserts in 18S rDNAs of zooxanthellate tridacnids and cardiids, as well as in azooxanthellate cardiids, when aligned with 18S rDNA sequences of *Limicolaria kambeul, Mytilus edulis, Placopecten magellanicus, Chlamys islandica, Spisula solidissima,* and *Tresus nuttali.* *, identical base sequence. Numbers at both ends of the sequence indicate the position of the base in the aligned sequences, which are available at the following URL:
http://www.mbl.edu/html/BB/home.BB.html

cardiid, proto-tridacnid, and pre-hippopus, before reaching the final tridacnid stage (Kawaguti, 1983b). This suggests that the tridacnid clams evolved from the ancestral cardiid clam. The present phylogenetic topologies (Figs. 1–3) are consistent with this idea.

Insertions in E10-1 in azooxanthellate cardiids are found in the variable region. The identities between these insertion sequences are relatively low, but they might appear after divergence from both of the zooxanthellate cardiids and tridacnids. In region 47, the insertions are restricted to tridacnid clams, except *T. gigas.* This insertion might appear after divergence between *T. gigas* and other *Tridacna* species.

Fossil records of Tridacnidae species are known from the Eocene (Stasek, 1961) and those of *Fragum* only from the Miocene (Keen, 1980). No *Corculum* fossils have been found before the Recent (Keen, 1980). More detailed molecular phylogenetic study, in combination with physiological and paleontological studies on the zooxanthellate and azooxanthellate bivalves, may give us insight into the evolution of symbiosis with zooxanthellae in bivalves.

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